INHIBITION OF FERTILIZATION IN ARBACIA BY BLOOD EXTRACTS

WILLIS E. PEQUEGNAT

Marine Biological Laboratory, Woods Hole, Massachusetts, and Department of Zoology, Pomona College, Claremont, California

From the time of publication of F. R. Lillie's paper (1914) on fertilization in Arbacia, some embryologists have maintained that the serum of Arbacia blood [1] provided an effective block to fertilization in this and a few other marine invertebrates. Lillie formulated the hypothesis that fertilization in Arbacia was actuated through the conjoining of certain constituents of egg and sperm by a substance called fertilizin, the presence of which in solution could be detected by the agglutinating action which it exerted upon sperm in aqueous suspension (see Tyler, 1948, for recent review of the subject). Asserting that filtered blood of Arbacia was capable of inhibiting fertilization while it did not prevent fertilizin from agglutinating sperm, Lillie linked this inhibitory action into his conception of the mechanism of fertilization by postulating that the serum-inhibitor prevented the uniting of fertilizin with the necessary constituent of the egg.

Oshima (1921) published the results of a few experiments which had motivated him to suggest that an external ("dermal") secretion was responsible for the inhibition observed by Lillie. That Oshima was not prepared to enter a complete denial of Lillie's conclusions is evidenced by his admitting that filtered blood was capable of exhibiting a weak though unpredictable inhibitory influence upon the fertilizability of the egg. Interestingly enough the degree of inhibitory action considered weak by Oshima fell within the range certainly considered significant by Lillie. Furthermore, it is worthy of note that Lillie was not able to offer a satisfactory explanation of the fact that the potency of undiluted blood samples displayed degrees of inhibitory effectiveness varying from zero to one hundred per cent. Nonetheless, largely through the influence of E. E. Just, little or no attention was paid to Oshima's suggestions by the majority of interested embryologists, except, perhaps, for Harvey (1939).

Apparently critical data confirming Lillie's conclusions were brought forth by Just (1922), who, at the same time, brushed aside Oshima's contraindications without any statement that he had attempted to repeat the latter's experiments. Also, Just stated that the most plausible explanation of Oshima's results would depend upon the presence of excretory or defecatory wastes in his solutions.

The matter rested at this point until the summer of 1946 when, at the suggestion of Dr. Albert Tyler, Richard L. Murtland, Albert H. Banner, and the present

[1] It has been convenient to use the word blood as a synonym of the term perivisceral fluid, even though strict interpretation may not warrant the practice. For present purposes the words serum and plasma are considered as literal equivalents when applied to Arbacia, since in this organism the clot is believed to be purely of cellular composition. Since Lillie and Just had previously used serum to denote the material obtained from whole blood by clotting, filtering, or centrifugation, I have followed this choice entirely for the virtue of consistency.

author [2] collaborated briefly in repeating a few of Lillie's experiments. Becoming interested in the mechanism of this inhibitory action, I carried on alone during the latter part of the summer of 1946 and returned to Woods Hole in the summer of 1947 to proceed with the same problem.

Although at the time I was unaware of Oshima's publication, I undertook to verify Lillie's observations before proceeding to a study of the *modus operandi* of the inhibitor. Following Lillie's techniques as closely as possible, I obtained results which corroborated his. Thus, I was convinced that his conclusion to the effect that Arbacia serum contained a factor capable of inhibiting fertilization was valid. But when, during the summer of 1947, I introduced techniques of collecting blood designed to yield uncontaminated samples, I obtained results which revealed that Lillie's original description of the source of the inhibitor must be modified. During the course of this study, it was found that serum samples removed by syringe so that they were uncontaminated with drainage from the exterior of the test did not possess inhibitory activity. Furthermore, it was found that sea water extracts from the tests of intact Arbacia were not capable of inhibiting fertilization. Thus, it became evident that some step in Lillie's technique, which I had followed previously in obtaining corroboratory results, was responsible for the appearance of the inhibitor in the serum samples. Chosen as the most likely cause was the fact that prior to opening the perivisceral cavity both Lillie and Just rinsed their urchins in tap water, presumably to kill any sperm present on the test which would otherwise fertilize samples of eggs. I had noticed that the application of tap water, even when followed by a sea water rinse within a few seconds, caused a yellow substance to appear in the excess water draining from the test of the as yet intact animal. A detailed study of this phenomenon revealed that this yellow exudate was capable of inhibiting fertilization. Additional experiments revealed that the immediate source of this inhibitor was to be found in certain granules or cells located in the tube feet and a few other organs. And, contrary to the findings of Lillie and Just, the ultimate source of the inhibitor was found to be some of the blood cells found in the perivisceral fluid. The present paper gives the details of these experiments.

Method

The sea-urchin *Arbacia punctulata* was the principal animal used in these experiments. Perivisceral fluid was removed from Arbacia by methods designed both to permit contamination from the outside and by methods devised to prevent such contamination. In addition, various techniques were devised which might supply information relative to the ultimate source of the inhibitor. Also, one significant step not used by previous workers was added to the routine handling of all samples. Having noted that the pH of sea-urchin blood was lower than that of sea water, and since this in itself may interfere with fertilization (Tyler and Scheer, 1937), it was decided that the pH of all samples should be adjusted to that of sea water. Moreover, in order to obviate any modification of results arising from undue concentrations of egg or sperm secretions, all samples containing gametes were discarded.

In most experiments one drop each of eggs and sperms were introduced into 2 cc. of fluid, be it sea water or extract, in Syracuse dishes. In each instance the

[2] Three members of the 1946 Embryology Class of the Marine Biological Laboratory.

eggs from a single female were used for each series of experiments. The average concentration of eggs in suspension was found by actual count to be between 2200 and 2500 eggs per drop. Fresh sperm suspensions were used for fertilizations and were made by diluting one drop of dry sperm with the equivalent of 99 drops of sea water. The various fluids were tested in serial two-fold dilutions of 2 cc. down to 64-fold. Determinations of the percentage of fertilization were made by actual counts under low magnification from three to five hours after insemination. The practice of first scanning the dish and then counting between four and five hundred eggs along two diameters was followed consistently.

Most of the experiments with differently prepared fluids were run simultaneously, as is indicated by similar dates in the tables.

Experiments

Series I. This experiment was carried out essentially as outlined by Lillie and Just, as follows: (1) Arbacia rinsed in tap water for a few seconds, shaken and rinsed in filtered sea water; (2) animals permitted to drain, cut made with scissors around peristome, and fluid drained into Syracuse dish; (3) clot permitted to form, checked for presence of gametes, then filtered; and finally (4) samples were centrifuged lightly and the pH adjusted to that of sea water. The fluid was then used undiluted, or diluted as described above. In each instance the fluid obtained in this way had a yellowish tinge.

This series of experiments, involving a total of 42 animals, was repeated seven times between July 8 and August 2, 1947. From the results tabulated in the left half of Table I it can be observed that this solution was effective in blocking fertilization when used undiluted. An average of approximately 3 per cent fertilizations was obtained in undiluted fluids as compared to nearly 100 per cent fertilizations of eggs in sea water controls. A summary of part of Lillie's work (1914), in which undiluted fluid collected from 50 Arbacia in the same manner and within the above dates was used, gives an average of 50 per cent fertilizations, as compared to 97 per cent in the sea water controls. The apartness of our results can be explained in part by the fact that his samples were used individually, while in my experiments fluid from all individuals was pooled before being tested. For example, Lillie's data show that the serum obtained by him from one individual contained no inhibitor, while the serum from another contained enough to inhibit all eggs tested. This would yield an average of 50 per cent inhibition. On the other hand, if these two samples had been pooled before being tested, it is possible that sufficient inhibitor would be present in the mixture to give complete inhibition. A comparison of the effects of diluting the serum show this conjecture to be valid. Thus, the percentage of fertilization increased when the fluid collected on August 2 was diluted, as follows:

Percentage serum (in sea water)	100	50	25	12.5	6.2
Percentage fertilization	0	16	68	99	100

In this experiment I obtained an average of 32 per cent inhibition with a 25 per cent solution of serum, and Lillie's data show that he obtained 30 per cent inhibition when using a 20 per cent solution of serum obtained in the same manner.

Series II. The tap water rinse was eliminated in this experiment; otherwise all procedures were the same as those outlined in Series I. Again, fluid from a total of 42 animals was tested on seven occasions between July 10 and August 3, 1947. The fluid in each case was clear, not yellow.

The results, as tabulated in the right half of Table I, offer a marked contrast to those of Series I. There is no significant difference between the percentages of fertilizations obtained from eggs inseminated in undiluted serum and those inseminated in sea water (both yielding approximately 99 per cent fertilizations). Hence it became apparent that the application of tap water was linked in some manner with the appearance of the inhibitor. Additional experiments were performed in order to determine whether it was being liberated into the serum from within the animal or from the outside.

TABLE I

Serum obtained by cutting peristome

With tap water rinse (Series I)					Without tap water rinse (Series II)				
July	pH	Adjusted pH	Percentage fertilizations		July	pH	Adjusted pH	Percentage fertilizations	
			Serum (100%)	Sea water				Serum (100%)	Sea water
8	7.5	8.0	0	100	10	7.6	8.0	100	100
10	7.4	8.0	0.5	100	12	7.6	7.9	99	99
12	7.6	8.0	0	99	14	7.7	8.0	100	100
14	7.6	8.0	0	100	21	7.5	7.9	100	100
26	7.2	8.0	10	100	26	7.5	8.0	95	100
27	7.5	8.0	5	99					
Aug.					Aug.				
2	7.5	8.0	0	100	2	7.6	8.0	100	100
					3	7.0	8.0	100	100

Series III. In order to circumvent contamination of serum with external drainage, the fluid was withdrawn by means of a 10 cc. syringe equipped with a No. 22 gauge hypodermic needle. The needle was introduced into the perivisceral cavity through the peristome about 3 mm. from Aristotle's lantern. Care had to be exercised to keep the needle from penetrating the gonads and to prevent the plunger from crushing blood cells when fluid was expelled from the syringe. When the proper depth of penetration had been determined, the needle was ensheathed with rubber tubing long enough to stop it at the desired level. All animals used in this experiment were rinsed momentarily in tap water and then in sea water prior to withdrawing the fluid. Subsequently, the fluid was prepared and used exactly as described previously. The fluid in all cases was clear, not yellow.

Eight experiments were performed with serum collected in this manner between July 8 and August 3. The data from this series are tabulated in Table II. An average of 99.1 per cent fertilizations was obtained from eggs fertilized in all undiluted samples of this serum. This evidence, when coupled with the results of Series I, indicated that the inhibitor evoked by tap water drained into the perivisceral fluid from the outside when the latter was collected by cutting the peristome. Moreover, there seems little reason for doubting that these facts explain

the large range of variation in potency of inhibitor recorded both by Lillie and Just, because the amount of drainage in their samples would have varied inversely with the time elapsing between rinsing the animals and withdrawing the fluid, and directly with the time required to drain each animal.

Thus far the following facts have been ascertained: (1) that the inhibitor of fertilization is not found in the serum of the intact Arbacia; (2) that tap water causes the inhibitor to appear in samples of serum collected by the method of Lillie and Just (Series I); and (3) that the inhibitor so evoked comes from the outside of the animal.

It is important to note that when inhibition has been observed up to this point perivisceral fluid plus external drainage have been in solution together. Further experiments were performed to reveal whether this complex was necessary for

TABLE II

Serum withdrawn by syringe after tap water rinse

(SERIES III)

July	pH	Adjusted pH	Percentage of fertilizations	
			Serum (100%)	Sea water
8	7.5	7.9	100	100
10	7.6	8.0	100	100
12	7.6	7.9	98	99
14	7.7	8.0	99.5	100
17	7.9	7.9	100	100
26	7.5	7.9	95	100
Aug.				
2	7.5	8.0	100	100
3	7.0	8.0	100	100

inhibition, or whether the yellow drainage alone was sufficient to cause inhibition of fertilization. The fourth series of experiments was devoted to this problem.

Series IV. Several Arbacia were washed in filtered sea water to remove debris and wastes, then rinsed under the tap for fifteen seconds, and finally submerged briefly in filtered sea water to correct hypotonicity. After being shaken the animals were placed edgewise in glass funnels fitted with moistened filter paper, and the yellow drainage collected in centrifuge tubes. An equal number of control animals was subjected to the same treatment except that no tap water rinse was administered. Material from any animals that proceeded to defecate or shed gametes was discarded. The animals were left in the funnels a maximum of 20 minutes, or until draining ceased. The filtrate was centrifuged, although no visible separation occurred, and the pH adjusted to that of sea water. The clear, yellow fluid was then used as before.

This experiment was repeated seven times between July 10 and August 2, using a total of 68 animals. The results shown in Table III indicate that this material alone is sufficient to bring about inhibition of fertilization. In only two instances was fertilization obtained in this material when undiluted, and then only an average of 2 per cent of the eggs were activated. These data show also that

TABLE III

(SERIES IV)

Yellow fluid obtained from the test by draining after tap water rinse

July	pH	Adjusted pH	Percentage of fertilizations							
			Fluid dilutions (per cent)						Rinse control 100	Sea water control 100
			100	50	25	12	6	3		
10	7.6	8.1	1	—	—	—	—	—	97	99
12	7.8	8.0	0	—	—	—	—	—	100	100
14	7.9	8.0	0	—	—	—	—	—	100	100
17	7.8	8.1	0	0	0	0	17	100	96	97
19	7.6	7.9	0	0	0	0	0	—	95	98
*27	7.5	7.0	0	0	0	0	0	0		
27	7.5	8.0	3	2	3	3	3	99	100	100
27	7.5	9.0	21	29	10	10	21	84		
Aug. 2	7.6	8.0	0	0	0	0	1	5	100	100

* The experiment of July 27 was carried out at three pH's. The inhibitor's effectiveness is apparently reduced at pH 9. Note also an increase of effectiveness with moderate dilution; this occurred at other pH's as well.

these solutions effectively blocked fertilization in dilutions ranging from 50 to 3 per cent by volume. As a check some of this material was mixed in varying amounts with perivisceral fluid which alone had no effect on fertilization. As was anticipated, the previously impotent serum now became inhibitory to fertilization in proportion to the amount of yellow drainage added (Table IV). No appreciable difference in the inhibitor's activity could be detected between that diluted with sea water and that diluted in serum. Since the liquid draining from the control animals (those not rinsed in tap water) was found to be ineffective in blocking fertilization, it appears unlikely that this inhibition is due to the presence of soluble wastes, at least as proposed by Just in answer to Oshima's report.

TABLE IV

(SERIES IV)

Inhibitor mixed with impotent serum

Percentage of components in mixture		Percentage fertilizations	
Serum	Inhibitor	Mixture	Sea water
95	5	98	100
90	10	78	—
85	15	24	—
80	20	2	—
75	25	0	—
70	30	0	—
50	50	0	100

Series V. Much more potent solutions of inhibitor were obtained by (1) placing urchins directly into distilled water to depths not exceeding the greatest circumference of the shell and permitting them to remain 15 minutes, and (2) adjusting the osmotic value of the solution with sea water concentrated by evaporation. Control animals were placed in sea water to soak for the same period of time as the test animals. The osmotic values of these test solutions were checked by comparing the diameters of test eggs with those of the controls. No significant variations were observed. In addition one control was composed of equal volumes of distilled water and sea water concentrated to half its original volume by evaporation.

Although this experiment was run on several occasions, only one will be described in detail since all were essentially the same. On July 31 half a dozen Arbacia were placed in succession into 40 cc. of distilled water and permitted to remain approximately 5 minutes each. Six control animals were placed into the same volume of sea water in the same manner. The distilled water was immediately colored yellow, while the sea water remained clear and colorless throughout. After filtration, centrifugation, and adjustment of osmotic value, 62 cc. of yellow fluid were obtained. This obviously represents a much greater dilution per animal than in previous experiments. But despite this fact this solution prevented fertilization completely in serial dilutions down to 1 per cent. The sea water in which control animals had stood gave 100 per cent fertilizations, as did the other control solution.

Series VI. In order to narrow down the locus of origin of the inhibitor, five animals were cut into halves along the oral-aboral axis. All internal organs were removed and the inside of the tests scrubbed in sea water with a brush. Following this the sectioned tests were soaked for one hour in sea water, which was not discolored in the process. Then the tests were rinsed for a few seconds in tap water and sea water, and permitted to drain into a clean finger bowl. The drainage was yellow. The spines on the tests were still moving when the fluid was removed after one hour. The pH was adjusted from 7.6 to 8.0, and the material tested. The following results were obtained:

Percentage of extract	100	50	25	12	6	3
Percentage of fertilizations	3	6	3	3	92	100

When the same tests were again rinsed in tap water and sea water and permitted to drain, only 2 cc. of fluid were obtained. When used undiluted this second drainage gave 10 per cent aberrant cleavages. Controls gave 98 and 99 per cent fertilizations, respectively. These results provided additional evidence that some external structure was the source of the inhibitor.

Series VII. A study of individual tube feet under the microscope revealed a layer of closely packed, yellow granules or cells just beneath the outer epithelium. These granules maintained their integrity while immersed in sea water. But when the sea water was replaced with tap water, all traces of yellow material disappeared from within the feet. Simultaneously with this disappearance, the water around the feet was colored bright yellow. It is important perhaps to note that this material diffused through the outer epithelium and did not pass into the lumen of the foot. Tests run on this yellow material proved that it possessed the property

of inhibiting fertilization in a manner similar to that observed previously. One experiment will be described in greater detail.

Part of the tube feet from the oral hemispheres of four Arbacia were removed. This was done by letting the animal attach to a glass plate and then pulling the plate away. The tube feet were soaked in several rinses of sea water, covered momentarily with tap water and then placed into 4 cc. of clean sea water. The latter water was immediately colored yellow. After five minutes the supernatant fluid was decanted and its volume noted to be 5 cc. It was then filtered and the pH raised from 7.6 to 8.0. It is worthy of note that this extract was brighter yellow than that obtained by placing the whole animal in distilled water. The color of the extract deepened to a certain extent when the pH was elevated. Interestingly enough I found subsequently that it became colorless at pH 4 and below, and a darker yellow at pH 8 and above.

This tube-foot extract proved to be very effective at blocking fertilization. In concentrations running from 100 to 12 per cent no fertilizations occurred, and only 1 per cent of the eggs was fertilized in dishes containing as little as 3 per cent extract in sea water. This degree of effectiveness is made more remarkable by the fact that because of the dilution intrinsic to the method of extraction the extract represented only 20 per cent by volume of the inhibitor solution that exuded from the tube feet. Hence, the 12 per cent solution in the series of 2 cc. dilutions would actually contain a maximum of 0.025 cc. of inhibitor, or approximately 1.25×10^{-5} cc. per egg.

Because the color of this extract was not the same shade as that obtained when intact animals were used, I searched for other sources. It was found that spines gave forth a small amount of inhibitor, but only from their bases where epithelium was to be found. The bodies and tips of the spines, which in many instances had no fleshy covering, gave up a purplish substance which had no significant effect on fertilization. When this substance was mixed in small amounts with the extract from tube feet, however, the latter assumed the color of the extract from the intact animals.

Series VIII. Further work revealed that the inhibitor was carried by at least one type of amoebocyte found in the perivisceral fluid. Blood was removed by cutting the peristome, but attention is called to the fact that the animals were not rinsed in tap water. And, instead of filtering the blood as before, the plasma or serum was separated from the cells by light centrifugation and then decanted into clean flasks. At this time, an equal volume of sea water was added to the clot in the tube and the two mixed by shaking and rapid centrifugation. Whereas the plasma was colorless, the supernatant sea water solution was the same bright yellow as the extract from the tube feet. The pH of the two solutions offered an additional point of contrast. Whereas the pH of the plasma was 7, that of the yellow extract was 6.3, despite the fact that the sea water was pH 7.9 at the time of its addition to the clot. It is suggested that this depression of pH was caused by the liberation of the acid contents of the colorless amoebocytes. Before being tested, both solutions were brought up to pH 8.

A further contrast of properties of these two solutions was observed when they were tested: the percentage of fertilization in solutions of plasma equalled that of the controls, in this case 99 per cent; the yellow extract, however, permitted no

fertilizations when used undiluted. A two-fold dilution of the extract permitted only 10 per cent of the eggs to be fertilized. These results showed clearly that the inhibitor was carried by certain blood cells, and in such a manner that it did not normally pass from them into the plasma. Attempts to isolate the specific type or types of blood cells that carried the inhibitor were nullified by the fact that no practical method was devised for preventing the blood from clotting. The methods usually employed to prevent clotting of vertebrate blood were found to be of no value. Nonetheless, it was possible to observe microscopically that upon cytolysis the amoebocytes with yellow spherules (for classification of blood cells, see Kindred, 1926) gave up a yellow substance which upon addition of acid became decolorized as does a solution of the inhibitor. Two additional observations also serve to link the inhibitor obtained by methods described previously with that obtained directly from the blood cells.

It was possible to demonstrate that the potency of inhibitor extracts obtained from blood clots of animals that had been soaked previously in distilled water was less than that obtained from untreated animals. For example, blood was removed by syringe from six animals which had been used just previously for obtaining inhibitor by soaking in distilled water (after the method of Series V). The blood was then centrifuged, the plasma decanted and replaced by sea water, and the mixture shaken and centrifuged rapidly. These solutions were tested with the result that no fertilizations were permitted in the extract obtained by soaking in distilled water; 50 per cent fertilizations were obtained from the undiluted sea water extract of the clot; and 100 per cent fertilizations were obtained in the plasma and sea water controls.

The reciprocal of the above was also found to be true, viz., that animals from which all possible perivisceral fluid had been removed by syringe produced weaker solutions of inhibitor obtained by application of tap water (after the method of Series IV). I took six animals from which pervisceral fluid had just been removed by syringe, and rinsed them briefly in tap water and sea water, and then placed them into funnels from which the drainage was collected. This drainage permitted an average of 32 per cent fertilizations when used undiluted.

All of the observations made in this series of experiments lend some support to the opinion that the yellow granules observed in the tube feet may actually be yellow amoebocytes that are free to move between tube feet and the perivisceral cavity.

Series IX. The following experiment was devised to show whether or not the inhibitory effect of blood extracts upon eggs was reversible. One drop of eggs was placed into 2 cc. of undiluted inhibitor solution contained in each of six Syracuse dishes. After insemination the dishes were placed in running sea water on the water table. No fertilizations resulted in any of the dishes. At the end of two hours, the inhibitor solution was pipetted from one of the dishes and the eggs washed twice in fresh sea water and then reinseminated. Eggs in the remaining dishes were handled in the same manner 4, 8, 12, 14 and 24 hours after being introduced into the inhibitor solution. A series of six control dishes contained approximately the same number of eggs in 2 cc. of sea water; these were fertilized in series after the same intervals of time. The results are tabulated in Table V. Although there are some indications that some eggs were damaged by standing in the inhibitor solution, there is definite evidence that this blocking of fertilization is reversible.

TABLE V

(SERIES IX)

Fertilization of blocked eggs after washing

Exposure to inhibitor (hours)	Percentage fertilizations		
	Before washing	After washing	Controls
2	0	97	100
4	0	92	98
8	0	95	96
12	0	*90	94
14	0	†99	98
24	0	90	93

* About 30 per cent exhibited polyspermy.
† About 10 per cent aberrant cleavages.

Series X. Eggs that were observed to develop in various dilutions of inhibitor had indicated that post-fertilization developmental processes were not appreciably affected, but one experiment was run to test this point more effectively. Eggs were inseminated in sea water and transferred as quickly as possible into dishes containing undiluted inhibitor. Subsequent examinations revealed no significant differences between those samples of eggs that were placed in inhibitor and those that remained in the sea water. The zygotes were kept in the original inhibitor solution until they had reached the swimming stage. At this time they were transferred to sea water and carried on to the pluteus stage. Two observations were made during this time: (1) there were some indications that the rate of development was retarded slightly by the inhibitor, and (2) that the plutei developing from inhibitor-treated zygotes were smaller than the controls. These observations were not investigated further.

DISCUSSION

The results obtained from Series II and III of experiments refute the validity of the conclusion of Lillie and Just that the serum of Arbacia blood normally contains an inhibitor to fertilization. Although Series VIII showed that the inhibitor was carried by certain blood cells, the results of Series II indicated that the inhibitor did not leave the blood cells to enter the serum. In fact, the inhibitor was obtained from the cells in Series VIII only after vigorous shaking and rapid centrifugation. Since it has been shown that the inhibitor can be evoked by the application of tap water to the outside of the animal, it may be concluded that the inhibitor observed by them entered their samples from the outside of their urchins as a result of the application of tap water. Furthermore, the supposed variations in potency of the inhibitor reported by Lillie and Just were shown to be illusory by the results of Series I and III.

Certainly the property of inhibition is not of itself particularly interesting for no doubt a large number of substances could be used to inhibit fertilization in Arbacia, but most of them would very likely be inimical in one way or another to the gametes. Therefore, the fact that eggs appear to be fundamentally unharmed

by exposures to this natural inhibitor ranging from a few seconds to many hours serves to heighten one's interest. It has been shown that eggs which have remained blocked up to 24 hours in this inhibitor can be fertilized, provided they are washed thoroughly in sea water and reinseminated. Moreover, Just (1922) reported that he obtained development in blocked eggs (inseminated in inhibitor solution) without reinsemination, so long as the eggs were washed within two hours after fertilization.

Except for a slight depression of the rate of development, this inhibitor exerted no appreciable influence upon post-fertilization changes in the egg. Eggs that were fertilized at one instant and transferred immediately to inhibitor proceeded to develop into normal blastulae; yet when eggs were introduced into potent inhibitor and sperm added as quickly as possible blocking was complete. This latter observation supports the hypothesis that the inhibitor acts at the surface of the egg. Other evidence may be brought to bear on this point.

The inhibitor appeared to remove part of the egg's jelly layer, in proportion to concentration or to the duration of exposure. Inferred at first from the observation that eggs tended during the period of contact with inhibitor to aggregate more compactly than eggs in sea water, this conclusion was strengthened by the addition of dilute solutions of Janus Green B. Furthermore, the jelly layer of Chaetopterus eggs exhibited a marked affinity for the Arbacia inhibitor by staining a deep yellow during exposure, but the jelly layer was not removed by it. It is interesting to note also that this yellow cast was not removed by subsequent washings. Because the inhibitor obtained from Arbacia was observed to prevent fertilization of Chaetopterus eggs, it is unfortunate that no attempt was made to determine their fertilizability after washing. This might well have revealed whether the inhibitor itself is yellow or is only associated with the pigment in solution. While referring to associated species, it is appropriate to record that the Arbacia inhibitor does suppress fertilization in the sand-dollar, Echinarachnius parma. This fact was reported by all previous workers. In addition, Just (1923) stated that the blood of this sand-dollar blocked the fertilization of its eggs. It is possible, however, that this observation is subject to the same criticism herein advanced against his interpretation of the Arbacia inhibitor, because I observed that tap water evoked a similar response from Echinarachnius. Unfortunately, I could find no complete description of the method he used in obtaining this fluid.

Normal fertilization membranes were seldom observed on eggs that were fertilized in fresh sea water after prolonged exposures to the inhibitor. It is possible that this condition resulted from simple aging of the eggs. But in some instances no membranes could be observed even after the eggs began to cleave. That this was not tight membrane development is attested to by those extreme cases in which the blastomeres rounded up and were as easily separable as those of eggs treated with Ca-free sea water.

It was more difficult to observe definitive effects of the inhibitor on sperm. Little positive evidence as yet obtained rules out the possibility that the inhibitor acts directly upon the sperm. But this position could be rendered less tenable by several observations. In the first place, sperms appeared to be stimulated to greater activity when in the presence of inhibitor; and, secondly, they continued to move about inhibited eggs long after all evidence of motility of sperm had dis-

appeared in the controls. One point in this connection that might be of value in future work is the fact that the sperms which persisted in activity longest appeared to have lost their ability to attach to the egg. Thus, they moved about aimlessly among the eggs without attempting to penetrate. Finally, Just (1922) reported the actual penetration of sperms into the cortex and cytoplasm of blocked eggs. Presumably these were the sperms that were able to consummate fertilization when such blocked eggs were washed within the two-hour limit but not reinseminated.

Differences of opinion have arisen concerning the interaction, if any, between inhibitor and fertilizin. Lillie (1914) concluded that the effects of the inhibitor could be nullified by mixing it with fertilizin. One cannot question the data from which he drew this conclusion, but it appears to the present author that the method that he used to obtain neutralization of the inhibitor permits another interpretation of his data. In order to combine fertilizin with inhibitor, Lillie mixed serum and whole eggs in the ratio of two parts serum to one part eggs. Time intervening, the mixture was filtered and the filtrate tested for inhibitory activity. His data show that the untreated serum permitted only 0.5 per cent of the eggs tested to be fertilized, while the treated serum permitted 99.0 per cent of the eggs tested to be fertilized. Lillie concluded that the fertilizin had neutralized the inhibitor in the serum. But it is possible that little or no inhibitor was left in the filtrate. The basis for this interpretation is supplied by an experiment not previously described.

I mixed 4 cc. of inhibitor solution, obtained in the manner of Lillie, with 2 cc. of a suspension of eggs computed to contain approximately 0.75 cc. of sea water (Solution 1). Another 4 cc. of the same inhibitor were mixed with 0.75 cc. of sea water (Solution 2). A third solution was prepared by adding 2 cc. of strong egg-water (known to agglutinate sperm) with another 2 cc. of the inhibitor solution (Solution 3). After an interval of twenty minutes, all tubes were centrifuged lightly and 2 cc. samples were removed carefully from the top of each tube, and tested in the usual manner along with sea water controls. The following results were obtained: Solution 1 gave 75 per cent fertilization; Solutions 2 and 3 gave 0.0 per cent fertilizations; and 99 per cent of the eggs in the sea water controls were fertilized. These data suggested that the inhibitor combined in some manner with the eggs. Also, the fact that no fertilization occurred in the mixture of inhibitor and egg-water (Solution 3) supports the contention that the fertilizin does not neutralize the inhibitor, at least in the same sense of the word as used by Lillie.

The results of this experiment (particularly from Solution 1) provide a tentative explanation of the retarded activity of eggs that have stood in dilutions of inhibitor for some time after insemination. It is obvious that individual eggs in any sample are affected differentially by the inhibitor; otherwise there could be no explanation of the interesting fact that in dilutions of inhibitor some eggs are fertilized while others are not. Furthermore, one should recall that blocked eggs may be reversed to a state of fertilizability by soaking them in sea water. Therefore, it is possible that eggs which at first have the minimum of inhibitor necessary to prevent fertilization give this up slowly when the diffusion gradient has reversed, as the result of the greater affinity of other eggs for inhibitor. Just what conditions in or on the egg account for this differential reaction to inhibitor, I cannot say.

Although it appeared that fertilizin exerted no appreciable influence upon the

activity of the inhibitor, some evidence was obtained which indicated that a reverse influence did exist. On the one hand, as indicated in the introductory paragraph of this report, Lillie asserted that the inhibitor did not reduce the ability of fertilizin to agglutinate sperms. On the other hand, I found that concentrated solutions of inhibitor, when mixed in the ratio of 1 : 3 with egg-water capable of agglutinating sperm, would reduce the time required for reversal of agglutination. When these substances were mixed in equal parts, the egg-water lost its ability to bring about agglutination.

An interesting aspect of this problem is revealed by the close parallel between certain properties shared by the inhibitor and material extracted by Tyler (1940) from Arbacia eggs. Both of these materials are yellow; both appear to negate the sperm-agglutinating power of filtered egg suspension; both exhibit tendencies to cause clumping of eggs; and, under certain conditions, both reduce the fertilizability of eggs. The material extracted from eggs, however, produces a visible precipitation membrane on the egg's jelly layer; no membrane of this type has as yet been observed upon applying the blood inhibitor. Nonetheless, this parallelism between the properties of these two extracts is such that further study is indicated.

Summary

1. Whole perivisceral fluid (blood) of Arbacia contains a substance capable of inhibiting fertilization.

2. Contrary to the conclusions of previous investigators, this inhibitor is not normally present in the serum. Rather certain blood cells, particularly the amoebocytes with yellow spherules, are the ultimate source of the inhibitor.

3. The inhibitor believed by Lillie and Just to be found in the serum of Arbacia blood actually entered their samples as a contaminant from the outside of their animals.

4. The external application of tap water causes the inhibitor to appear in the drainage from the test. Under these conditions the inhibitor emanates from yellow bodies found in the hypodermis of the tube feet.

5. The supposedly variable potency of inhibitor reported by previous workers can be explained by the technique used in obtaining samples, and the methods used in testing its strength. In reality former workers were testing inhibitor in varying dilutions rather than testing the potency of a standard amount of inhibitor.

6. This inhibitor does react with the egg's jelly layer and can modify the fertilization membrane in proportion to concentration and duration of exposure. Eggs that are inhibited for short intervals of time (1–4 hours) can be fertilized and will develop normally (i.e. with membranes, etc.), provided they are rinsed thoroughly in fresh sea water.

7. Fertilizin is believed to have little influence on the activity of the inhibitor beyond a simple dilution effect. On the other hand, the sperm-agglutinating power of fertilizin-bearing solutions can be reduced or nullified by the addition of sufficient inhibitor.

8. It is suggested that this blood inhibitor may be related to an egg-agglutinin extracted from the egg itself.

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